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14. ABSTRACT

The pathways that normally mediate AR ubiquitylation and degradation remain to be established. Newly synthesized AR associates with an HSP90 chaperone complex, and an HSP90 associated E3 ubiquitin ligase (CHIP) may mediate the polyubiquitylation and proteasome degradation of AR that fails to fold appropriately and bind ligand. However, there are clearly additional cytoplasmic and/or nuclear ubiquitin ligases that regulate the normal turnover and degradation of the liganded AR. Indeed, multiple ubiquitin ligases have been reported to interact with AR and regulate its transcriptional activities and/or degradation. Moreover, previous studies using overexpressed ubiquitin ligases and AR site-directed mutagenesis have implicated a number of lysines as possible sites for ubiquitylation. However, the physiological sites and corresponding ubiquitin ligase pathways that regulate AR degradation remain to be defined, and whether AR antagonists or other agents can enhance these pathway to enhance AR degradation remains to be determined. Our studies have identified sevral novel ubiquitylation sites on the AR. Moreover, functional studies of one such site. K911, indicate that ubiquitylation at this site may negatively regulate AR binding to chromatin and transcriptional activity. Studies are underway to identify how ubiquitylation at this site is regulated and its precise functional consequences.

15. SUBJECT TERMS

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INTRODUCTION

The androgen receptor (AR) plays a central role in prostate cancer (PCa) development and progression. Most patients initially respond to androgen deprivation therapy (ADT, surgical or medical castration), but the tumors inevitably recur despite castrate levels of androgen (castration-resistant prostate cancer, CRPC). AR expression is generally increased in CRPC, and one mechanism driving its activity is intratumoral synthesis of androgens from adrenal gland derived precursors or *de novo* from cholesterol. This activity can be suppressed by drugs including abiraterone (which inhibits the enzyme CYP17A1 required for androgen synthesis) or by the direct AR antagonist enzalutamide, and both abiraterone and enzalutamide are now approved for treatment of CRPC. However, patients who respond to these agents generally relapse within a year, and AR appears to still be contributing to the growth of these relapsed tumors.

Therefore, there remains a critical need to develop therapeutic approaches that can further suppress AR activity in advanced CRPC, and in particular therapies that may enhance AR degradation. However, pathways that normally mediate the ubiquitin dependent or independent degradation of AR, which might be targeted therapeutically, remain to be established. AR degradation is presumed to be preceded by AR polyubiquitylation, but the ubiquitylation of specific sites on AR that drive this process and the relevant ubiquitin ligases remain to be firmly established. CHIP (C-terminus of Hsc70 interacting protein) is a ubiquitin ligase that may mediate the polyubiquitylation of the unliganded AR, possibly through an interaction with the AR N-terminus, but the role of CHIP in AR degradation remains to be clarified (Adachi et al., 2007; Cardozo et al., 2003; Morishima et al., 2008; Rees et al., 2006). AR also may be ubiquitylated by other ubiquitin ligases (including MDM2, UBCH7, TAF1, RNF6, TRIM68, and PIRH2), although many of these may mediate monoubiquitylation and/or function to modulate (enhance or suppress) the transcriptional activity of the liganded AR (Chymkowitch et al., 2010; Gaughan et al., 2005; Lin et al., 2002; Tavassoli et al., 2010; Xu et al., 2009).

Ubiquitin ligases also may recognize distinct structural features of the liganded AR, such as AKT mediated phosphorylation of S213 or CDK7 mediated phosphorylation of S515, which have been reported to stimulate MDM2 binding (Chymkowitch et al., 2010; Lin et al., 2002), versus the HSP90 associated unliganded AR that may be targeted for degradation due to exposure of hydrophobic surfaces. Therefore, ubiquitylation regulates AR functions in addition to the degradation of unliganded AR. One of our objectives is to identify such pathways mediating the turnover and degradation of liganded nuclear AR, as these may possibly be exploited therapeutically through the development of selective AR antagonists that activate these pathways.

BODY

Aim 1. Identify and characterize ubiquitylation sites on AR

Subaim 1a. Identify ubiquitylation sites associated with degradation of the unliganded AR. We outlined in our last progress report our initial results using liquid chromatography followed by tandem mass spectrometry (LC-MS/MS) to identify ubiquitylation sites on AR in cells cultured in androgen depleted medium or medium with androgen (see subaim 1b). For these studies we focused on VCaP cells, as they have an amplified wild-type AR, and thereby express high levels of AR protein. Our previous results as well as further data are shown in the updated Table 1. Limited peptide coverage (~65% overall) and low stoichiometry remain as problems, so we clearly cannot exclude ubiquitylation at additional sites.

Subaim 1b. Identify ubiquitylation sites associated with the liganded AR. We have carried out similar analyses from androgen (DHT) stimulated VCaP cells. The combined results from a series of LC-MS/MS analyses of DHT stimulated cells are also shown in Table 1. As we are particularly interested in the ubiquitylation status of AR on chromatin, we have also carried out studies wherein we covalently crosslink AR to chromatin prior to cell lysis, as is done for chromatin immunoprecipitation. Our preliminary results indicate that this crosslinking markedly increases the amount of AR we detect in the chromatin fraction. However, our initial attempts at analyzing this material by LC-MS/MS have not been successful, and further efforts are

underway.

Condition	Appox.MW (kDa)	Ubiquitylation	Methylation	Acetylation
CSS +MG132	MW >125 kDa	K911 (1/1) K837 (1/1) K826 (1/3) K823 (1/2) K639 (1/1)	K659 (1/8) (1/5) K639 (1/1) K634 (1/1) (2/2) R264 (1/6) R129 (1/4) R20 (3/3)	
	MW ~110 kDa	K911 (1/2)	K823 (1/4) K659 (2/8) (1/7) K639 (1/2) K634 (1/2) R407 (1/3) K318 (1/3) K313 (1/7) R129 (1/2) R101 (1/6) R31 (1/4) R20 (2/2) R13 (1/1)	K826 (1/2)
DHT (10 nM) +MG132	MW >125 kDa	K718 (1/1) K862 (1/1) K911 (1/1)	R129 (1/3) R20 (1/1)	
	MW ~110 kDa		K639 (1/1) R20 (1/1)	K313 (1/5) K222 (3/6)

Table 1. AR post-translation modifications detected by LC-MS/MS in androgen-depleted and androgen-stimulated VCaP cells. VCaP cells were cultured for 24 hours in androgen medium with androgen depleted serum (charcoal/dextran stripped serum, CSS), followed by 4 hours treatment with proteasome inhibitor (MG132), minus or plus DHT. AR was then isolated from whole cell lysates and run on SDS-PAGE, followed by excision of area ~100 Kd (mono or possibly oligo-ubiquitylated AR) and >125 kD. Gel slices were then digested with trypsin, and analyzed by LC-MS/MS. Numbers in parentheses indicate the number of times each modification was detected in one preparation.

Subaim 1c. Determine the functional significance of ubiquitylation sites. To identify possible functions for the identified ubiquitylation sites, we have used site directed mutagenesis to convert each lysine to arginine. Data in the previous progress report indicated that ubiquitylation at lysines 639, 837, and 911 can contribute to AR degradation under low androgen conditions. To further assess for functional significance, we used reporter gene assays to examine effects of site directed mutants on AR transcriptional activity. Lentiviruses encoding AR wild-type or mutants were used to establish stable transfectants in PC3 cells (no endogenous AR), with each line expressing comparable amounts of AR (not shown). Transfection with an ARE regulated Luciferase reporter gene then showed that only the K911A mutant AR had increased activity (~2-fold) (Fig. 1A).

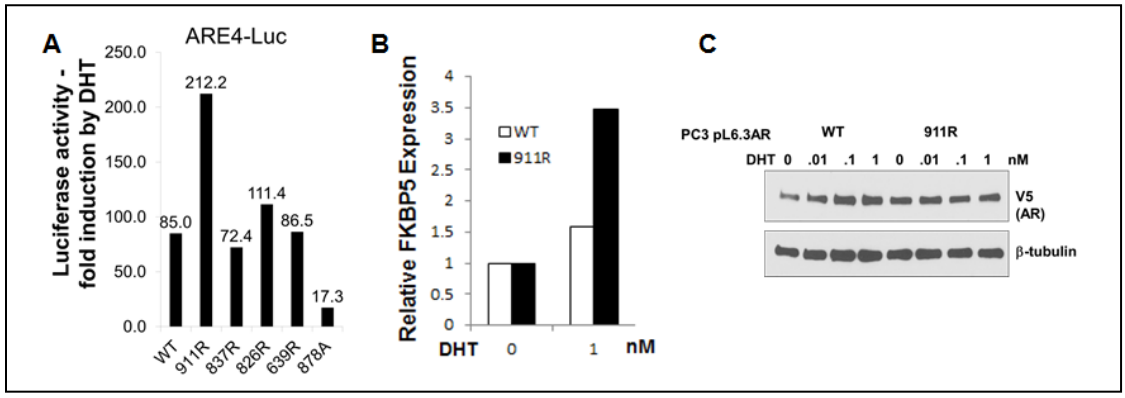

Figure 1. Functional assays for AR lysine residues. (A) PC3 cells were stably transduced with WT or mutant AR and then transiently transfected with ARE4-Firelfy Luciferase and control Renilla Luciferase expression vectors. Replicate samples were then treated for 24 hours with vehicle versus 10 nM DHT and ratio of Firefly vs Renilla Luciferase measured, and fold induction (DHT/vehicle) was determined. (B) PC3 cells stably transduced with WT or K911R mutant AR were treated with vehicle or 1 nM DHT for 24 hours and expression of endogenous AR regulated FKBP5 gene expression were determined (vehicle normalized to 1). (C) PC3 cells stably transduced with WT or K911R mutant AR were treated with vehicle or DHT (.01, 0.1, or 1 nM) for 24 hours and AR protein expression was assessed by immunoblotting (β-tubulin was used as protein loading control)

We then examined regulation of an endogenous AR regulated gene, FKBP5, by the stably expressed AR WT versus K911R mutant. As shown in figure 1B, the K911R mutant had increased activity in response to 1 nM DHT. Finally, while expression of the WT AR was increased by DHT (reflecting increased degradation of the unliganded AR), the stability of the K911R mutant AR was not increased by DHT (Fig. 1C). These findings indicated that the K911R mutant had increased stability and transcriptional activity.

The unliganded AR is primarily in the cytoplasm, and in response to androgen it rapidly accumulates in the nucleus and associates with chromatin. We therefore next examined whether the K911R mutation had an effect on AR cytoplasmic versus nuclear localization. As expected, prior to addition of DHT the WT AR was isolated primarily in the cytoplasmic fraction (Fig. 2A). Compared to the WT AR, there was more of the K911R AR in the nucleus and associated with chromatin in the absence of added DHT. Moreover, DHT more robustly stimulated chromatin binding by the K911R mutant AR (Fig. 2A). Consistent with these results, by chromatin immunoprecpitation (ChIP) we observed that increased levels of the K911R mutant AR were associated with the AR binding site in the FKBP5 gene (Fig. 2B). Taken together, these results suggest that ubiquitylation of K911 negatively regulates AR binding to chromatin and may enhance degradation of the chromatin bound AR. Further studies are now addressing this hypothesis.

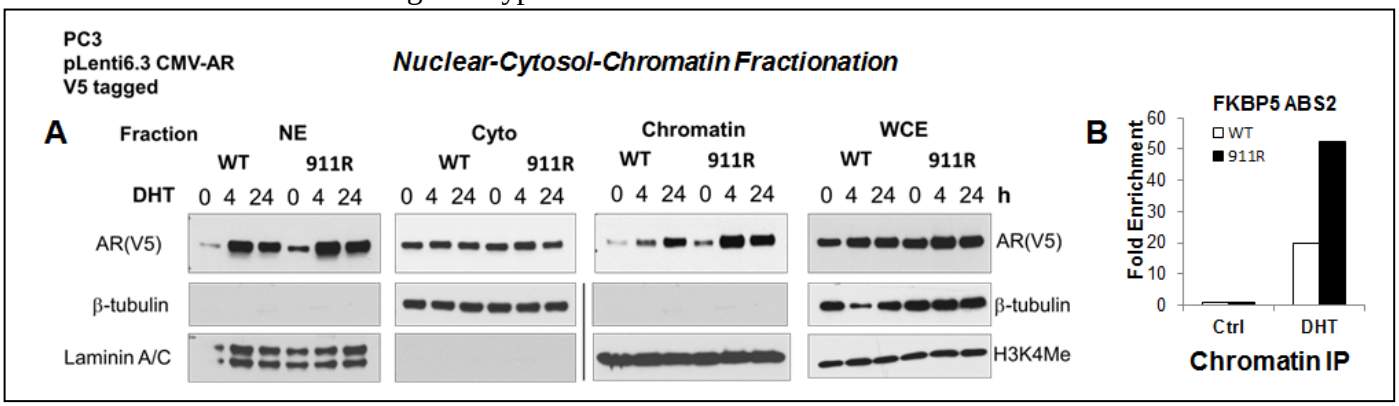

Figure 2. Effects of K911 on AR nuclear localization and chromatin binding. (A) PC3 cells stably expressing WT or K911R mutant AR were treated for with 10 nM DHT for 0, 4, or 24 hours. Cells were then lysed (whole cell lysate, WCE) and portions were fractionated into nuclear extracts (NE), cytoplasmic fraction (Cyto), and chromatin fraction (Chromatin). AR expression in ach fraction was then assessed by immunoblotting (V5 epitoipe tag on AR). (B) PC3 cells stably expressing WT or K911R mutant AR were treated with vehicle or 10 nM DHT for 4 hours. AR binding to the AR regulated enhancer in the FKBP5 gene was then assessed by ChIP.

Aim 2. Identify ubiquitin dependent mechanisms mediating nuclear and cytoplasmic AR degradation

a. Identify ubiquitin ligases mediating AR polyubiquitylation and degradation. As described in our previous progress report, used expression vectors for a series of E3 ubiquitin ligases to assess effects on AR after cotransfection into PC3 cells. In addition to decreased AR in response to SKP2 and MDM2, the FBL and TRCP E3 ligases decreased AR protein. The physiological significance of these latter E3 ligases is now being examined.

We had also previously reported that the protein phosphatase 1 catalytic subunit (PP1α) can enhance AR activity by dephosphorylating a site in the hinge region, Ser650 (Chen et al., 2009). Phosphorylation of this site was shown previously to enhance AR nuclear export (Gioeli et al., 2006), and we found that PP1α inhibition decreased nuclear expression of wild-type AR, but not an S650A mutant AR (Chen et al., 2009). However, in further studies we found that PP1α overexpression enhances the transcriptional activity of both the wild-type and S650A mutant AR, as assessed by co-transfection of AR, PP1α and an AR regulated reporter gene into HeLa cells or LNCaP PCa cells (Fig. 3A). Moreover, the effects of PP1α on the wild-type and S650A mutant AR were comparable (Fig. 3B). Together these results indicated that PP1α may regulate AR activity by one or more additional S650 independent mechanisms.

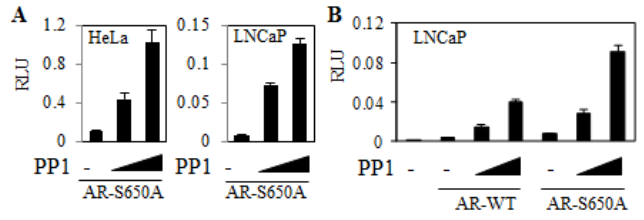

Figure 3. PP1α can increase AR activity of phospho-S650 dephosphorylation. (A) HeLa or LNCaP cells were co-transfected with AR-S650A mutant and PP1α, together with ARE4-Luc reporter and incubated overnight in androgen-depleted medium with DHT (10 nM). (B) LNCaP cells were co-transfected with ARE4-Luc reporter together without or with AR wild-type (WT) versus AR-S650A mutant and PP1α and overnight with 10 nM of DHT.

PP1α inhibition with tautomycin also decreased endogenous AR protein in both LNCaP and C4-2 cells, particularly in steroid-depleted medium without addition of androgen (dihydrotestosterone, DHT) (Fig. 4A and B). Significantly, tautomycin similarly decreased expression of the transiently transfected S650A mutant AR in HeLa cells (Fig. 4C), and the stably expressed S650A mutant AR in LNCaP cells (Fig. 4D). In both cases this effect was most pronounced in steroid-depleted medium in the absence of exogenous DHT. These results show that one mechanism through which PP1α can increase AR activity independently of S650 dephosphorylation is by increasing AR protein.

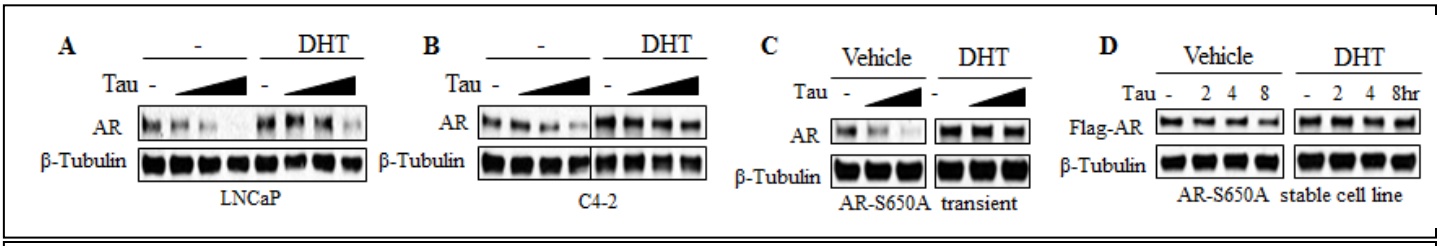

Figure 4. PP1α can increase AR independently of phospho-S650 dephosphorylation. (A) LNCaP and (B) C4-2 cells in androgen-depleted medium were treated overnight with tautomycin (Tau, 100, 200, and 400 nM) and androgen (10 nM DHT for LNCaP and 1 nM DHT for C4-2) as indicated and proteins were normalized for blotting. (C) HeLa cells were transfected with AR S650A mutant and (D) a LNCaP line was generated to stably express Flag-tagged AR-S650A construct. Cells in androgen-depleted medium were treated for indicated time points with androgen (10 nM of DHT) and tautomycin (Tau, 400 nM) and proteins were normalized for blotting.

To determine whether PP1α was increasing AR protein stability, we overexpressed PP1α and used cycloheximide to block new protein synthesis. As shown in figure 5A, PP1α overexpression increased the half-life of AR protein.

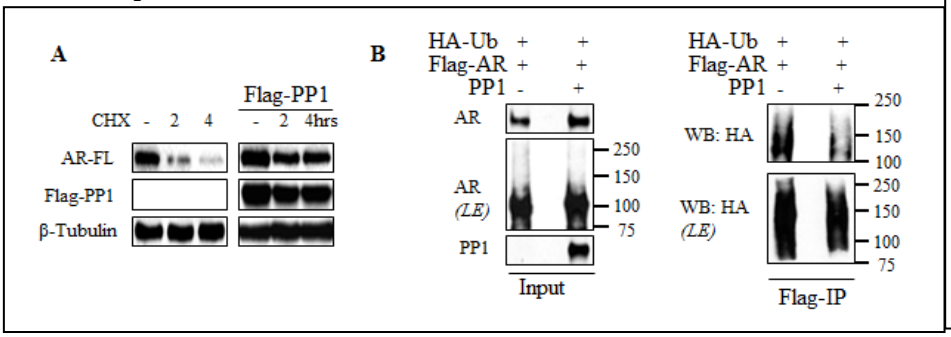

Figure 4. PP1α stabilizes AR by attenuating AR ubiquitylation. (A) HeLa cells in androgen-depleted medium were co-transfected with HA-AR full-length (FL) +/- Flag-PP1α, and treated with cycloheximide. (B) 293T cells were transfected with HA-Ub, Flag-AR, +/- PP1α, followed by culture in androgen-depleted medium.

To determine whether this effect was due to decreased AR ubiquitylation, we transfected cells with PP1α and epitope tagged ubiquitin. As expected, the input lanes showed that AR protein was increased by PP1α (Fig. 5B, left panel). We then immunoprecipitated AR and immunoblotted for the HA-tag on ubiquitin. Significantly, AR ubiquitylation was decreased by PP1α (Fig. 5B, right panel).

Several ubiquitin ligases, including MDM2 and SKP2, have been implicated as mediators of AR ubiquitylation and degradation (Chymkowitch et al., 2011; Gaughan et al., 2005; Li et al., 2014; Lin et al., 2002). MDM2 degradation can be enhanced by phosphorylation at multiple sites including S395, and previous reports indicate that PP1α can decrease the degradation of MDM2 by dephosphorylating this site (Lee et al., 2007). However, MDM2 activity can also be enhanced by growth factor stimulated phosphorylation of sites including S166 and S186 (Zhou et al., 2001). Effects of PP1α on SKP2 have not been addressed previously. Therefore, we next assessed whether PP1α may dephosphorylate and inactivate these ubiquitin ligases to attenuate AR ubiquitylation and degradation.

Consistent with previous reports, AR protein expression was decreased by cotransfection of MDM2 or SKP2 (Fig. 5A). Cotransfection of PP1α resulted in a dramatic increase in the levels of transfected MDM2 and SKP2, which appears to be primarily due to increased transcription and translation from the expression vectors rather than decreased protein degradation. However, this may also in part reflect PP1α mediated inactivation of MDM2 and SKP2 enzymatic activities, as this would suppress autoubiquitylation and degradation (Fang et al., 2000; Xu et al., 2007). In either case, despite the dramatic increases in MDM2 and SKP2, there were no increases in the ability of these proteins to suppress AR expression, indicating that their activity was markedly impaired by PP1α (Fig. 5A).

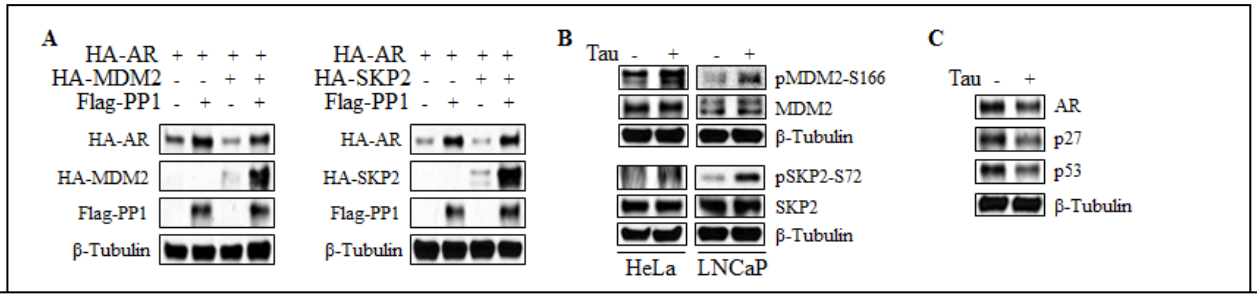

Figure 5: PP1α can dephosphorylate AR degrading E3 ligases. A. HeLa cells were co-transfected with HA-AR, Flag-PP1α and HA-tagged MDM2 or SKP2 as indicated. Cells were then incubated for overnight in androgen-depleted medium for blotting. B. HeLa and LNCaP cells in androgen-depleted medium were treated for 3 hrs with 200 nM of tautomycin, followed by protein normalization for blotting. C. LNCaP cells in androgen-depleted medium were treated for 3 hrs with tautomycin as indicated. Total proteins were normalized for blotting.

To determine whether endogenous PP1α may regulate the phosphorylation and activity of these ubiquitin ligases, we treated HeLa and LNCaP cells with tautomycin and assessed for effects on endogenous MDM2 and SKP2. We did not observe clear increases in MDM2 or SKP2 in either cell line (Fig. 5B). However, tautomycin increased MDM2 phosphorylation at S166, which has been reported to enhance MDM2 activity (Zhou et al., 2001). Similarly, tautomycin increased SKP2 phosphorylation at S72, a site that may be phosphorylated by AKT and lead to increased stability and activity (Lin et al., 2009; Rodier et al., 2008). Consistent with increased activity of MDM2 and SKP2, tautomycin treatment decreased levels of their respective substrates, p53 and p27 (Fig. 5C). Taken together these findings indicate that PP1α inhibition may increase AR ubiquitylation and degradation by increasing the activity of one or more AR degrading ubiquitin ligases.

b. Identify AR degradation pathways that may be enhanced by AR antagonists. We previously reported that the AR antagonist galeterone could enhance degradation of the T878A mutant AR (Yu et al., 2014). Studies

are pending to assess whether this is mediated through a specific pathway and site.

KEY RESEARCH ACCOMPLISHMENTS

-identification of sites on AR that are ubiquitylated under steroid-depleted conditions
-identification of sites on AR that are ubiquitylated in the presence of androgen
-generation and preliminary characterization of panel of ARs with mutations at each ubiquitylation site
-discovery that K911 site is involved with chromatin binding and transcriptional activity
-discovery that PP1α can suppress AR degradation mediated by ubiquitin ligases

REPORTABLE OUTCOMES

A manuscript describing the effects of PP1α on AR ubiquitylation and degradation is now in press (Liu et al., Protein Phosphatase 1 Suppresses Androgen Receptor Ubiquitylation and Degradation, Oncotarget, in press).

CONCLUSIONS

AR undergoes ubiquitylation at a series of sites that have not been identified or characterized previously. Our recent data indicate that ubiquitylation at K911 plays an important role in the turnover of AR bound to chromatin. The precise mechanisms regulating ubiquitylation at this site and its functional consequences are a focus of further studies.

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